COPPER ENZYMES IN ISOLATED CHLOROPLASTS.
POLYPHENOLOXIDASE IN *BETA VULGARIS*

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(WITH EIGHT FIGURES)

The chloroplast, as the seat of chlorophyll pigments in plants, occupies a unique position in the economy of the green cell. In recent years there has been a renewed interest in the reactions and properties of chloroplasts as a result of the work of HILL (11, 12) and HILL and SCARISBRICK (13, 14) who demonstrated that the reaction characteristic of photosynthesis in green plants, the evolution of oxygen, occurs in appreciable quantities in isolated chloroplasts under the influence of light and in the presence of suitable oxidants (2, 7, 8, 26).

In the course of an investigation of oxygen evolution by isolated chloroplasts it was deemed important to explore their enzymatic composition. Of special interest were considered enzymes capable of participating in oxidation-reduction reactions, and more particularly, those localized principally, if not entirely, in the chloroplasts. This paper presents evidence that a copper enzyme, polyphenoloxidase (otherwise known as tyrosinase or catecholase), is localized in the chloroplasts of spinach beet (chard), *Beta vul-garis.*

Methods

GROWING OF PLANTS.—Seeds of spinach beet were germinated and grown in sand in flower pots in a greenhouse, heated to prevent the temperature from falling below freezing in winter. The pots were watered with a nutrient solution of the following composition (made with local tap water): KNO_3—0.01M; $Ca(NO_3)_2$—0.002M; $NH_4H_2PO_4$—0.002M; $MgSO_4$ —0.002M; and A4, a combination of the micronutrients Mn, B, Ca, Zn

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(1). Iron was supplied with each application of the nutrient solution at the rate of 0.5 cc. of 0.5 per cent. ferrous sulphate solution to a liter of nutrient solution. The nutrient solution was applied at variable intervals depending on the weather: in the more favorable periods approximately every other day. Following five successive applications of nutrient solution, the cultures were thoroughly leached with tap water to remove excess salt, just prior to the next application of nutrient solution.

CHLOROPLAST PREPARATIONS.—The chloroplast material used throughout consisted not of intact chloroplasts but rather of chloroplast fragments which were obtained by the preparatory technique to be presently described. Preparations of this type are sometimes referred to as ''grana''. If that term be appropriately reserved for the naturally occurring dense bodies in chloroplasts, as recently demonstrated with the electron microscope by GRANICK & PORTER (10), the chloroplast fragments used in this investigation may be regarded as aggregates of grana embedded in the matrix or ''stroma''.

The chloroplast fragments were prepared by a procedure similar to that of ARONOFF (2). Leaves were picked from plants grown in sand cultures and placed in cold tap water. After a thorough washing in running cold water, they were put in moisture-proof pliofilm bags and kept from one half to several hours in a refrigerator. The turgid leaves were torn by hand into small shreds and ground for three minutes in a Waring Blendor with either M/15 potassium phosphate buffer or glass distilled water. About 50 grams of laminae (the midrib and large veins were discarded) were blended with 60 cc. of fluid. The speed of the Blendor was regulated by means of a variable voltage transformer to avoid foaming.

The Blendor mash was strained through cheesecloth and immediately filtered through a small plug of absorbent cotton placed in a glass funnel. The residue was discarded and the filtrate was centrifuged in an angle centrifuge for 1 minute at approximately 3000 r.p.m. to remove intact chloroplasts and such larger particles as still remained after straining through cheesecloth and filtering through cotton. The supernatant fluid from the brief centrifugation was a dark green fluid containing, as disclosed by microscopic examination, chloroplast fragments with few intact chloroplasts. The residue which included the intact chloroplasts was discarded. The supernatant was decanted and centrifuged for 20 minutes at about 4° C. in an International Refrigerated Centrifuge at a speed corresponding to approximately 25,000 g.

The high speed centrifugation brought about a sedimentation of the chloroplast fragments, leaving a pale yellowish-green supernatant which contained only a minute amount of chlorophyll. The supernatant, henceforth designated as the cytoplasmic fluid, was decanted. The residue was rinsed quickly in M/15 phosphate buffer at pH 6.5 to remove the adhering cytoplasmic fluid, and then resuspended in a small volume of M/15 phos-

phate buffered at pH 6.5 with the aid of a glass stirring rod or a small glass spatula and filtered again through a small wad of absorbent cotton. The last operation was combined with gentle pressing, by means of a glass rod, of the chloroplast suspension against the wad of cotton—a procedure found effective in breaking up lumps. The resuspended chloroplast fragments which will henceforth be designated as the chloroplast suspension, contained between forty and fifty times as much chlorophyll as the cytoplasmic fluid.

DETERMINATION OF CHLOROPHYLL.—The procedure for chlorophyll determination was based on the work of MacKinney (21) on the absorption of light by aqueous acetone (80%) extracts of chlorophyll. The concentrations of chlorophyll a and b were determined by measuring in a 10 mm. cell, the density of 80% acetone chlorophyll extracts with a Beckman spectrophotometer at 663 and 645 mμ and setting up simultaneous equations using the specific absorption coefficients for chlorophyll a and b as given by MacKinney (21).

(i) $D_{663} = 82.04 \, C_a + 9.27 \, C_b$

(ii) $D_{645} = 16.75 \, C_a + 45.6 \, C_b$

where C_a, C_b are grams per liter of chlorophyll a and b respectively, and D the density values at the respective wavelengths as obtained on the Beckman spectrophotometer. From equation (ii):

(iii) $C_a = \dfrac{D_{645} - 45.6 \, C_b}{16.75}$, substituting in (i) and solving for C_b we obtain

(iv) $C_b = 0.0229 \, D_{645} - 0.00468 \, D_{663}$; substituting this expression for C_b in (ii) and solving for C_a:

(v) $C_a = 0.0127 \, D_{663} - 0.00269 \, D_{645}$

Hence total chlorophyll, C, in grams per liter:

(vi) $C = C_a + C_b = 0.0202 \, D_{645} + 0.00802 \, D_{663}$ or expressing total chlorophyll, C, in terms of milligrams per liter:

(vii) $C = 20.2 \, D_{645} + 8.02 \, D_{663}$

To provide a rough check for the determination of total chlorophyll the light absorption data for chlorophyll a and b (21) were plotted and the curves were found to intersect at $\lambda = 652 \, m\mu$. By extrapolation, the value of the specific absorption coefficient for this wavelength was found to be 34.5. Another equation was therefore set up for total chlorophyll:

(viii) $D_{652} = 34.5 \, C + 34.5 \, C_b = 34.5 \, (C_a + C_b)$

hence   (ix) $C = (C_a + C_b) = \dfrac{D_{652}}{34.5}$

or expressed as mgs. per liter:

(x) $C = \dfrac{D_{652} \times 1000}{34.5}$

The following example will illustrate the procedure used for chlorophyll determinations; 0.5 cc. of the chloroplast suspension was shaken in a glass stoppered volumetric cylinder with 4.5 cc. of water and 20 cc. of acetone. The suspension was filtered and the density values for the respective wave-

lengths were determined on a Beckman spectrophotometer to be:

$$D_{645} = 0.187$$
$$D_{663} = 0.506$$
$$D_{652} = 0.275$$

Substituting the first two values into equation (vii) and multiplying by the dilution factor:

$$C = (20.2 \times 0.187 + 8.02 \times 0.506) \times \frac{25}{1000 \times 0.5} = 0.39 \text{ mgs. chlorophyll/cc.}$$

Similarly, by substituting for D_{652} in equation (x):

$$C = \frac{0.275 \times 1000}{34.5} \times \frac{25}{1000 \times 0.5} = 0.399 \text{ mgs. chlorophyll/cc.}$$

The example given illustrates the generally good agreement between the two values obtained for total chlorophyll concentration. It is clear, however, that equation (vii), which is based on the selection of more favorable wave lengths for precise measurement, is more trustworthy than equation (x).

The size of the aliquot used for chlorophyll determination was governed by the chlorophyll content. Generally 0.5 cc. of the chloroplast suspension or 5 cc. of the cytoplasmic fluid in 25 cc. of 80 per cent. acetone were found convenient.

For the determination of enzymatic activity generally 2 cc. of either the chloroplast suspension or the cytoplasmic fluid was placed in each reaction vessel. In the case of the chloroplast suspension this corresponded to a chlorophyll content of between 0.7 and 2.0 mgs.

MEASUREMENTS OF POLYPHENOLOXIDASE ACTIVITY.—All measurements of enzymatic activity were made manometrically at 20° C. using conical vessels of approximately 16 cc. volume. Catechol was used in the estimation of polyphenoloxidase activity, not as a substrate, however, but as a catalyst in the oxidation of ascorbic acid. The use of small quantities of catechol in the measurement of polyphenoloxidase activity was demonstrated by KUBOWITZ (18) in the oxidation of hexose monophosphate in the presence of its dehydrogenase and coenzyme II. KEILIN and MANN (16) have shown that although polyphenoloxidase does not oxidize ascorbic acid directly, the oxidation proceeds very rapidly in the presence of small amounts of catechol. This principle was also used by MILLER and DAWSON (22) in developing the chronometric method for measuring polyphenoloxidase activity. The catechol is enzymatically oxidized to the o-quinone by atmospheric oxygen; the o-quinone in turn is almost instantaneously reduced back to catechol by the ascorbic acid. The reaction comes to completion when all of the ascorbic acid has been oxidized.

The substrate mixture used consisted of 0.02 mgs. catechol and from 1.2 to 2.4 mgs. of ascorbic acid. The catechol was introduced to the side-arm and tipped into the main vessel at zero time. The total volume of the reaction mixture was 3 cc. Except as noted otherwise, the gas phase

was air; to absorb CO_2, the center well contained 0.2 cc. of 10% KOH and a small strip of filter paper. In measuring cyanide inhibition the center well fluid consisted of a mixture of KCN and KOH in the proportions given by ROBBIE (25). The reaction mixture was prepared and made to volume with M/15 phosphate buffer of pH 6.5.
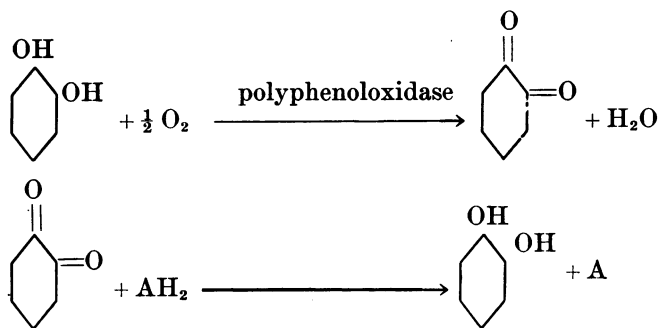
where AH_2 and A stand for ascorbic and dehydroascorbic acid respectively.

Half of the bottom of the constant temperature bath was made of plate glass. In testing the effect of light on the reversibility of carbon monoxide inhibition the reaction vessels were illuminated from below by a 500 watt condensed filament bulb placed in a specially designed metal reflector. The light intensity in the bath as measured by a Weston lightmeter was approximately 16,000 lux.

Results

LOCALIZATION OF POLYPHENOLOXIDASE ACTIVITY.—In a preliminary experiment it was found that the addition of catechol to a crude leaf mash of spinach beet plant was followed by a rapid oxygen uptake. This suggested the presence of a polyphenoloxidase in the leaves and it became of interest to determine whether this enzyme is found in the chloroplasts or in the cytoplasmic fluid, obtained after the separation of chloroplasts and their fragments.

As stated earlier, the method selected for measuring polyphenoloxidase activity was the oxidation of ascorbic acid with the addition of catalytic amounts of catechol to act as an oxygen carrier: oxidizable by the enzyme and reducible by the ascorbic acid. There was thus at no time a high concentration of the possibly toxic o-quinone in the reaction mixture (18, 23). Curve II in figure 1 shows that the leaf mash was capable of oxidizing ascorbic acid at a slow rate without the addition of catechol. This oxidation was, however, greatly accelerated by the addition of 0.02 mgs. of catechol (Curve I, fig. 1).

Upon separation of the chloroplast fragments from the cytoplasm it was found that the polyphenoloxidase activity was localized in the chloro-

plasts (fig. 2). Rapid uptake of oxygen and oxidation of ascorbic acid
followed the addition of catechol to the chloroplast suspension (Curve I).
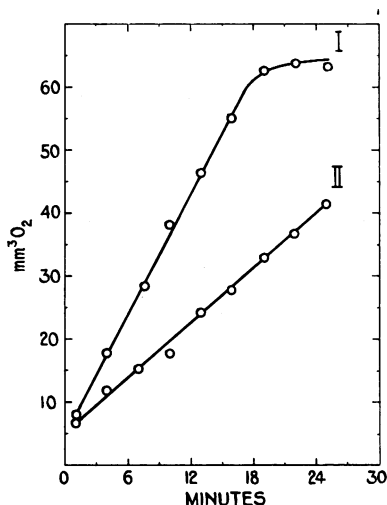Without the addition of catechol (Curve II) or with catechol but without

FIG. 1. Effect of added catechol on oxidation of ascorbic acid by leaf mash (super-
natant after 1 minute centrifugation as described under Methods). Curve I—0.02 mgs.
catechol in 0.2 cc. phosphate buffer tipped in at zero time to reaction mixture; Curve
II—no catechol added. Reaction mixture: 2 cc. of leaf mash containing 0.7 mgs. chloro-
phyll, 1.2 mgs. ascorbic acid in 0.6 cc. phosphate buffer, $M/15$ phosphate buffer (pH 6.5)
as needed to make a total volume of 3 cc. 0.2 cc. of 10% KOH and filter paper in
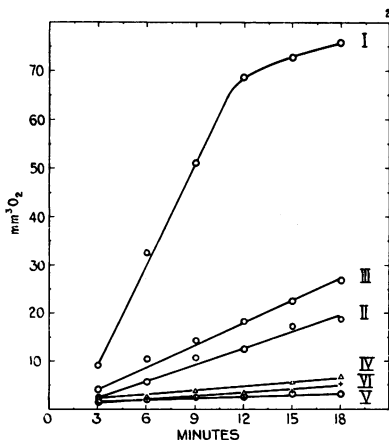center well.

FIG. 2. Polyphenoloxidase activity of chloroplast suspensions and cytoplasmic fluid.
Curve I—chloroplast suspension, catechol and ascorbic acid. Curve II—chloroplast sus-
pension and ascorbic acid but no catechol. Curve III—chloroplast suspension, catechol
but no ascorbic acid. Curve IV—cytoplasmic fluid, catechol and ascorbic acid. Curve
V—cytoplasmic fluid, ascorbic acid but no catechol. Curve VI—cytoplasmic fluid, cate-
chol but no ascorbic acid. Approximately 1 mg. of chlorophyll per vessel in chloroplast
suspensions and 0.02 mgs. of chlorophyll in cytoplasmic fluid. Conditions not specified
were the same as given in the legend of figure 1.

ascorbic acid (Curve III) the oxygen uptake was greatly reduced. Such residual enzymatic activity as was observed under these two latter conditions was probably due to substances naturally occurring in the chloroplasts which were capable of serving as substrates.

In contrast to the chloroplast suspensions, the addition of catechol, with (Curve IV) or without ascorbic acid (Curve VI) was without effect on the oxygen uptake by the cytoplasmic fluid. Nor did the addition of ascorbic acid alone (Curve V) result in an increased rate of oxidation by the cytoplasmic fluid. The polyphenoloxidase activity was thus found to be localized in the chloroplasts.
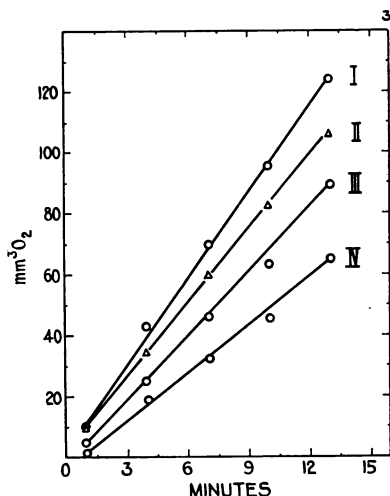

FIG. 3. Effect of cyanide on polyphenoloxidase activity of chloroplast suspensions. Curve I—control; Curve II—4.6×10^{-5}M CN; Curve III—10^{-4}M CN; Curve IV—4.6×10^{-4}M CN. 2 mgs. of ascorbic acid in all reaction mixtures. Center well contains KCN–KOH mixture. Conditions not specified were the same as given in the legend for Curve I of figure 1.

EFFECT OF INHIBITORS.—Consistent with the well established copper-protein nature of polyphenoloxidase (16, 18, 19), the activity of the enzyme in the chloroplast suspensions was inhibited by cyanide and carbon monoxide, the latter being insensitive to light (15). The enzyme was also inhibited by a more specific copper poison, sodium diethyldithiocarbamate (20). The effects of cyanide on the polyphenoloxidase activity of the chloroplast suspension are shown in figure 3, the inhibition by diethyldithiocarbamate in figure 4, and the inhibition by carbon monoxide in figure 5. As shown by Curve II of figure 5, light did not reverse the inhibition of enzyme activity by carbon monoxide.

IS THE LOCALIZATION OF POLYPHENOLOXIDASE ACTIVITY IN THE CHLOROPLASTS TO BE ATTRIBUTED TO PREPARATORY TECHNIQUE?—This question deserves careful consideration in attempts to identify the original locus of an enzyme found in the mash of leaves. The procedures used for disintegrating

leaves were necessarily drastic and resulted in disruption of cytoplasmic bodies including the chloroplasts. The persistence, however, of an enzyme in the resulting fragments, in this instance in the chloroplast suspension, and its absence from the cytoplasmic fluid, is under the circumstances regarded as strong presumptive evidence that the chloroplasts were the original site of the enzyme in the leaf. Conversely, were the enzyme to be found either partly or wholly in the cytoplasmic fluid, the possibility that it passed there following the fragmentation of chloroplasts could not have been easily excluded. Further experiments were carried out to test the conclusion about the localization of polyphenoloxidase in the chloro-
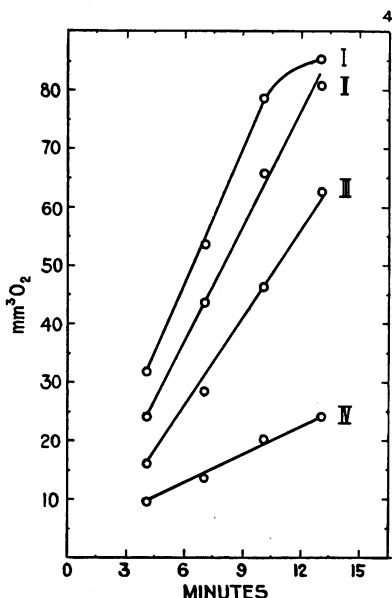

FIG. 4. Effect of sodium diethyldithiocarbamate (DDC) on polyphenoloxidase activity of chloroplast suspensions. Curve I—control; Curve II—10-4M DDC; Curve III —5 x 10-4M DDC; Curve IV—10-3M DDC. 1.5 mgs. of chlorophyll in all reaction mixtures. Conditions not specified were the same as given in the legend for Curve I of figure 1.

plasts, by modifying the experimental procedures in several respects, giving special attention to differences in technique which might have accounted for such divergent reports as were found in the literature.

a) The addition of potato polyphenoloxidase to leaf mash of spinach beet: the possibility was considered that if the polyphenoloxidase in spinach beet leaf were originally present in the cytoplasmic fluid, then the high-speed centrifugation used in separating the chloroplast fragments might have sedimented the enzyme as well. This point was tested by adding a crude potato polyphenoloxidase to the spinach beet leaf mash and then proceeding with the separation of the chloroplast fragments. The potato

polyphenoloxidase was prepared by a procedure similar to that of Szent-Györgyi as given by KEILIN (15).

A preparation of spinach beet leaf mash was divided into two batches. To one a solution of potato polyphenoloxidase in M/15 phosphate buffer (pH 6.5) was added, whereas the other batch served as a control. The
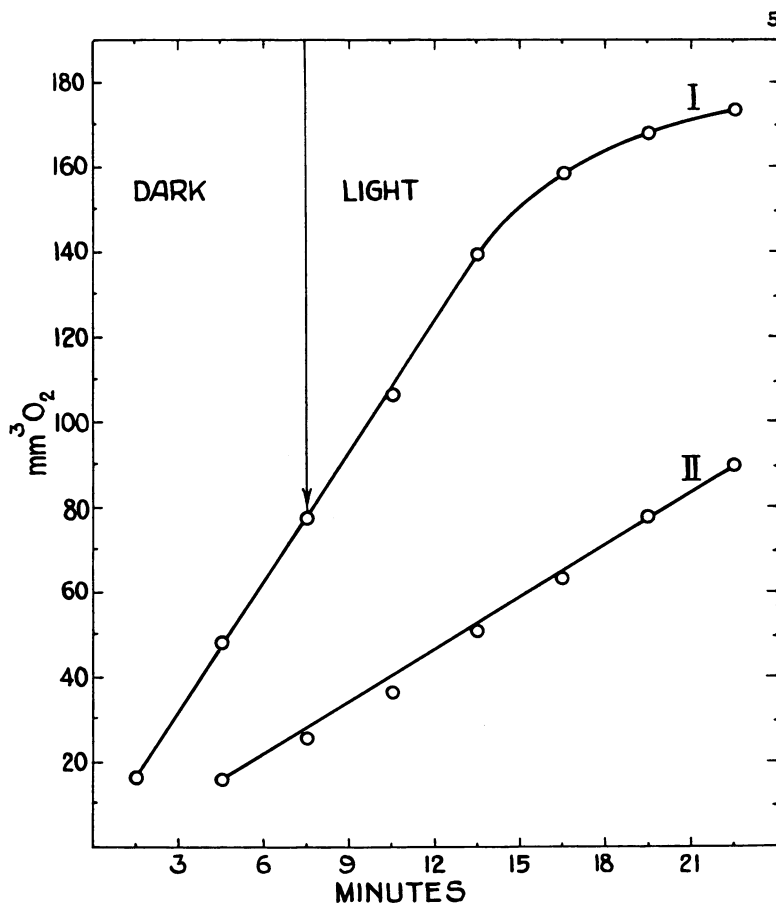

FIG. 5. Effect of carbon monoxide on polyphenoloxidase activity of chloroplast suspensions. Curve I—control in air; Curve II—90% CO—10% O$_2$. The turning on of light at 7.5 minutes did not reverse the inhibition by carbon monoxide. 2.2 mgs. of chlorophyll and 2.4 mgs. of ascorbic acid in each of the reaction mixtures. Conditions not specified were the same as given in the legend for Curve I of figure 1.

chloroplast fragments were then removed from the two batches of leaf mash, by high-speed centrifugation as previously described. The remaining cytoplasmic fluids were tested for polyphenoloxidase activity. The results, presented in figure 6, show that the added potato polyphenoloxidase was recovered in the cytoplasmic fluid. The activity of the polyphenoloxidase of the chloroplast suspension prepared from the batch of

leaf mash to which the potato enzyme was added was not enhanced. The conclusion was therefore drawn that the polyphenoloxidase activity of the chloroplast suspension was not to be attributed to sedimentation during the high-speed centrifugation.

b) Effect of pH of extracting buffer. WARBURG and LÜTTGENS (26), working with similar preparations from spinach and sugar beets, attributed about a quarter of the polyphenoloxidase activity of the crude green
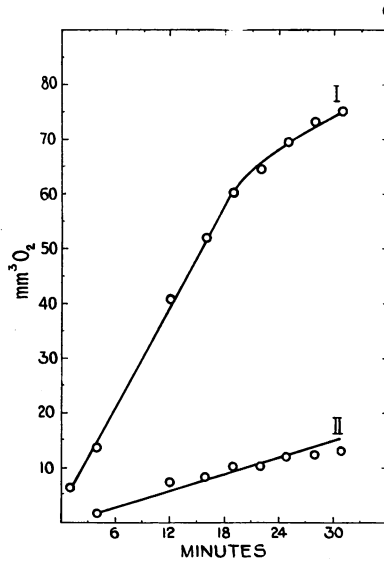

FIG. 6. Polyphenoloxidase activity of cytoplasmic fluids with and without the addition of a crude polyphenoloxidase preparation from potatoes. Curve I—potato polyphenoloxidase added; Curve II—control. Conditions not specified were the same as given in the legend of Curve I in figure 1.

leaf extract made at pH 6.5 to the chloroplast suspension and the remaining three quarters to the cytoplasmic fluid. These authors considered the pH of the extract important. Below pH 6.5, at pH 5 for example, they found that a major portion of the cytoplasmic polyphenoloxidase precipitated and in a subsequent centrifugation was recovered with the chloroplast fragments.

Although the preparations used in this investigation were uniformly made at pH 6.5 it was undertaken to test the effect of hydrogen ion concentration on the distribution of polyphenoloxidase by extracting the chloroplast suspension at the more alkaline pH 7.3. M/15 potassium phosphate buffer of pH 7.3 was used for grinding the leaves and resuspending the chloroplast fragments, the procedures otherwise remaining as previously described. The results presented in figure 7 show that, as with the former preparations, the polyphenoloxidase activity of leaf extracts made at pH 7.3 was also localized in the chloroplasts.

c) Effect of further disintegration of chloroplast suspensions. BONNER and WILDMAN (4) studied the respiration of spinach leaves and concluded that polyphenoloxidase is their terminal oxidase. Unlike the present findings, however, these authors reported the presence of polyphenoloxidase as confined to the cytoplasm rather than to the chloroplasts. The technique used by WILDMAN and BONNER (27) involved grinding the chloroplasts in a Charlotte Colloid mill with resultant disintegration of chloroplasts into chlorophyll-containing granules and non-chlorophyllous "stroma." These authors are aware that their conclusions as to the distribution of enzymes between chloroplasts and cytoplasm can be properly interpreted only with
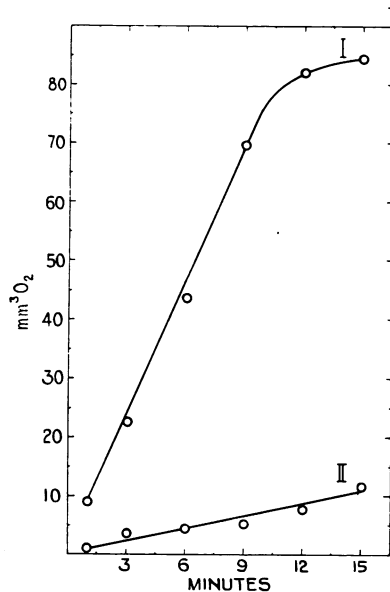

FIG. 7. The polyphenoloxidase activity of chloroplast suspension and cytoplasmic fluid prepared at an alkaline pH. The preparations and the reaction mixtures were made with potassium phosphate buffer (pH 7.3). Curve I—chloroplast suspension containing 1.5 mgs. chlorophyll; Curve II—cytoplasmic fluid. Conditions not specified are the same as given in the legend for Curve I of figure 1.

reference to their own manipulative technique. It was already noted that in the same plant, spinach, WARBURG and LÜTTGENS (26) found polyphenoloxidase both in chloroplast fragments and in cytoplasm. The spinach beet used in the present investigation is a chenopodiaceous plant related to sugar beet and spinach.

It seemed desirable in view of the results of BONNER and WILDMAN (4) to subject the already disrupted chloroplasts comprising the chloroplast suspensions used in this investigation to further disintegration. This was done by grinding them with glass beads on a Mickle tissue-disintegrating machine. The ground chloroplasts were then separated by high-speed centrifugation and resuspended in buffer as previously described. A micro-

scopic examination of the ground chloroplast suspension disclosed uniform round particles over 1 millimicron in diameter.

Figure 8 shows that this further subdivision of the chloroplast fragments failed to dislodge the polyphenoloxidase. As before, the enzyme
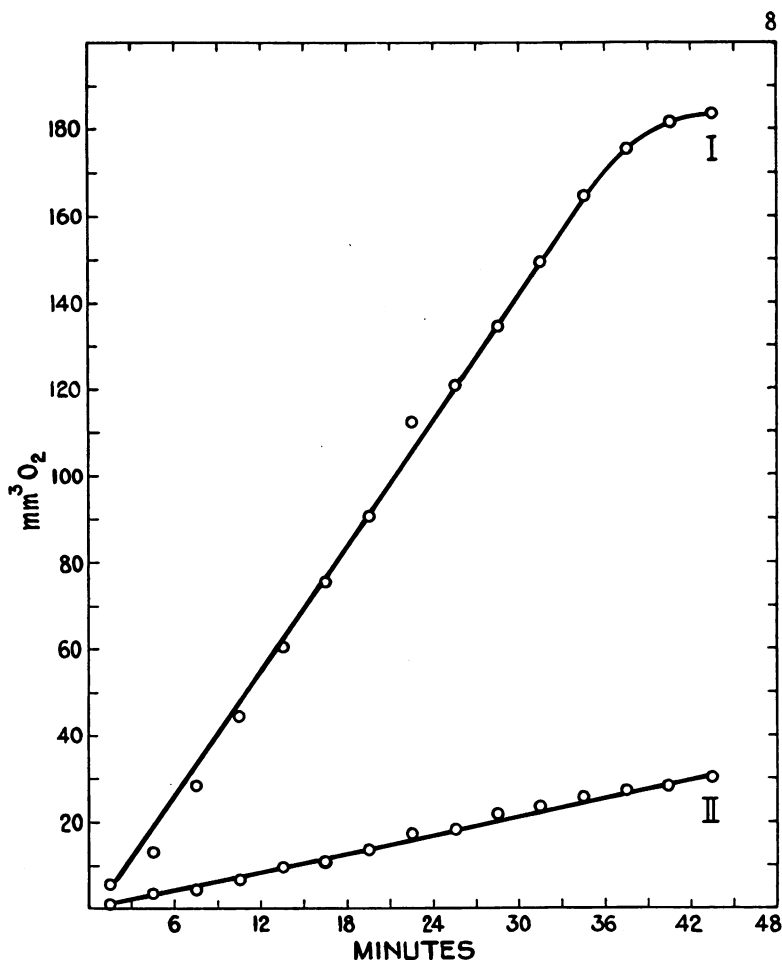

FIG. 8. Effect of further disintegration of chloroplasts on polyphenoloxidase activity. Curve I—disintegrated chloroplast suspension containing 0.8 mgs. of chlorophyll; Curve II—cytoplasmic fluid containing 0.02 mgs. of chlorophyll after removal of disintegrated chloroplast fragments. Reaction mixtures contained 2.4 mgs. ascorbic acid. Conditions not specified were the same as those given in the legend of Curve I in figure 1.

was found in the chloroplast suspension rather than in the cytoplasmic fluid.

TEST FOR LACCASE ACTIVITY OF CHLOROPLAST SUSPENSIONS AND CYTOPLASMIC FLUID.—The finding of polyphenoloxidase (an orthophenolase) activity in chloroplasts raised the question whether another copper protein enzyme,

laccase, which readily oxidizes paraphenols (17) occurs in leaf preparations. On *a priori* grounds this was considered unlikely: crude laccase oxidizes ascorbic acid directly (17) and it was already shown that neither the chloroplast suspensions nor the supernatant oxidized ascorbic acid directly at an appreciable rate. Furthermore, laccase, though capable of oxidizing catechol, is, unlike polyphenoloxidase, not inhibited by carbon monoxide (17). It was already shown, however, in figure 5, that catechol oxidation by chloroplast suspensions was inhibited by CO.

A preliminary test for laccase activity was made by adding 2 mgs. of *p*-phenylene diamine as a substrate to both the chloroplast suspension and the cytoplasmic fluid under conditions described in the legend of figure 1. The results indicated that no increased oxygen uptake resulted from the addition of *p*-phenylene diamine to either the chloroplast suspension or the cytoplasmic fluid.

Discussion

The presented data demonstrate the localization of the polyphenoloxidase of spinach beet leaves in the chloroplasts. The concentration of a copper-protein oxidase in a cellular body so intimately identified with the process of photosynthesis raises at once the question of the possible participation of this enzyme directly or indirectly in one or more of the biochemical reactions linked with photosynthesis. The answer to this question must await further experimentation.

In recent years there has been active interest in polyphenoloxidase as the terminal oxidase in the respiration of the potato tuber (3, 5, 6) and spinach leaves (4, 27). In Chlorella cells copper "poisons" have been found to inhibit both photosynthesis and respiration (9).

Whatever the ultimate position which may be assigned to polyphenoloxidase in the metabolism of plants, it appears certain that this enzyme cannot be of universal importance in the plant kingdom. This view is based on the findings of ONSLOW (24) that many, including some of the most common, mono- and dicotyledonous plants do not contain polyphenoloxidase. The elucidation, however, of its rôle in those plants in which the enzyme is found promises to lead to advances in comparative biochemistry through a search for its counterpart in other plants.

Summary

1. A green leaf extract of spinach beet (chard) was fractionated by high-speed centrifugation into a suspension of chloroplast fragments and a "cytoplasmic fluid" practically free of chlorophyll.

2. Polyphenoloxidase activity of the two fractions was tested by using catalytic amounts of catechol with ascorbic acid as substrate, and was found to be localized in the chloroplast suspension.

3. The localization of polyphenoloxidase in the chloroplast suspension was confirmed by using several variants in manipulative technique: ex-

traction of chloroplast fragments at a different pH, further disintegration of the chloroplast fragments, and the addition of a crude potato polyphenoloxidase to the leaf mash.

4. Consistent with the copper-protein nature of the enzyme, its activity in the chloroplasts was inhibited by cyanide, sodium diethyldithiocarbamate and carbon monoxide. The inhibition by carbon monoxide was not reversed by light.

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